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(54) Title: METHOD OF PROCESSING AND PRESERVING COLLAGEN BASED TISSUES

(57) Abstract

A process for the preserving collagen–based tissues involves procuring the collagen–based tissue; treating the tissue in a detergent solution; treating the tissue in an enzyme solution; treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the Maillard reaction and the subsequent formation of advanced glycosylation end products; treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via reactive oxidative species of molecules; treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the formation and propagation of molecular free radicals; treating the tissue in a cryopreservation solution; and cryopreserving the tissue. The process may be utlized to preserve several differing types of collagen based tissue including heart valve, vascular grafts including veins and arteries, umbilical vessels, nerve and nervous system tissue, dura, dermis and other similar collagen based tissues.

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METHOD OF PROCESSING AND PRESERVING COLLAGEN BASED TISSUES

This application claims priority of U.S. Provisional Patent Application No. 60/075,472 filed 20 February, 1998.

BACKGROUND OF THE INVENTION

Tissue and organ transplantation is a rapidly growing therapeutic field as a result of improvements in surgical procedures, advancements in immunosuppressive drugs and increased knowledge of graft/host interaction. Despite major advancements in this field, modern tissue transplantation remains associated with complications including inflammation, degradation, scarring, contracture, calcification (hardening), occlusion and rejection. There are numerous investigations underway directed toward the engineering of improved transplantable tissue grafts, however, it is generally believed in the industry that ideal implants have yet to be produced.

Autologous or self-derived human tissue is often used for transplant procedures. These procedures include coronary and peripheral vascular bypass surgeries, where a blood vessel, usually a vein, is harvested from some other area of the body and transplanted to correct obstructed blood flow through one or more critical arteries.

The motive for using autologous tissue for transplantation is based upon the concept that complications of immunorejection will be eliminated, resulting in enhanced conditions for graft survival. Unfortunately, however, other complications can ensue with autologous transplants. For example, significant damage can occur to several tissue components of transplanted veins during harvesting and prior to implantation. This damage can include mechanical contraction of the smooth muscle cells in the vein wall leading to loss of endothelium, and smooth muscle cell hypoxia and death. Hypoxic damage can result in the release of cellular lysosomes, enzymes which can cause significant damage to the extracellular matrix. Following implantation, such damage can lead to increased platelet adhesion, leukocyte and macrophage infiltration and subsequently further damage to the vessel wall. The end result of such damage is thrombosis and occlusion in the early post implant period. Even in the absence of such damage, transplanted autologous veins typically undergo thickening of the vessel wall and advancing

accelerated atherosclerosis leading to late occlusion. The exact cause of these phenomena is multifactorial and not fully elucidated. However, pre-implant hypoxia, mechanical damage caused during harvesting and handling of the tissue, and compliance mismatch of the vein in an arterial position of high blood pressure and flow rate have been implicated as being factors. Occlusion of transplanted veins can necessitate complicated repeat bypass procedures, with subsequent re-harvesting of additional autologous veins, or replacement with synthetic conduits or non-autologous vessels. As a result, the patient encounters a greater chance of severe post-operative morbidity and mortality.

When the supply of transplantable autologous tissues is depleted, or when there is no suitable autologous tissue available for transplant (e.g., heart valve replacement), then substitutes may be used, including man-made synthetic materials, animal-derived tissues and tissue products, or allogeneic human tissues donated from another individual (usually derived from cadavers). Man-made implant materials include synthetic polymers (e.g. polytetrafluroethylene (PTFE), DACRON™ and GORETEX™) sometimes formed into a tubular shape and used as a blood flow conduit for some peripheral arterial bypass procedures. When fashioned into a prosthetic heart valve, man made materials such as plastics and carbonized metals, may be utilized for aortic heart valve replacement procedures. Synthetic materials may be made with low immunogenicity but are subject to other limitations. In the case of mechanical heart valves, their hemodynamic characteristics necessitate life-long anticoagulant therapy. Synthetic vascular grafts, often used in above-the-knee peripheral vascular bypass procedures, are subjected to an even higher incidence of occlusion than autologous grafts. Indeed, the literature contains contraindications for the use of synthetic conduits for small diameter application (less than 6 mm diameter). In many cases, a preference is made for a biological implant which can be a processed animal tissue or allogeneic human tissue.

Animal tissues (bovine or porcine) treated with chemicals may be used as replacements for defective human heart valves and for vascular grafts. The concept in the chemical processing is to stabilize the structural protein and collagen matrix by cross-linking with glutaraldehyde or a similar cross-linking agent. Chemical treatment also masks histocompatibility antigens and other antigenic determinants. Thus, the human host should not recognize the implant as foreign

and reduces or eliminates a specific immunorejection response. However, despite the chemical treatment, the immune system may still recognize the implant as a foreign body resulting in encapsulation or overgrowth of the implant. In addition, glutaraldehyde-treated tissues often do not allow in-migration of host cells which are necessary for biochemical and biomechanical homeostasis. Gradually the glutaraldehyde treated tissue is reported to harden as a result of calcification and show functional impairment in 5-7 years. Glutaraldehyde-treated human and bovine umbilical veins have been used in the past for vascular bypass procedures, however, their use has been discontinued due to the unacceptable incidence of aneurysm formation and occlusion.

The use of allogeneic transplant tissues has been applied to heart valve replacement procedures, arterial bypass procedures, bone, cartilage, and ligament replacement procedures and to full-thickness wound treatment as a temporary dressing. The allogeneic tissue is used fresh, or may be cryopreserved, to maintain viability of cellular components. It is thought that the cellular components contain histocompatibility antigens, and are capable of eliciting an immune response from the host. In many cases, the patient receiving the allogeneic transplant undergoes immunosuppressive therapy. Despite this therapy, many allogeneic heart valves are reported to undergo an inflammatory response, and fail within 5-10 years. Allogenic cryopreserved saphenous veins have been shown in the literature to have a 72 % failure rate at one year after implantation.

Alternative processing methods have been developed by others that are intended to address the limitations of allogeneic and animal-derived transplant tissues. Freeze-drying is used routinely in the processing of allogeneic bone for transplantation. It has been found that the freeze-drying process results in a graft which elicits no significant rejection response as compared to fresh or cryopreserved allogeneic bone. It is believed that the freeze-dried bone following implant acts as a template, which is subsequently remodeled by the host. When the freeze-drying process has been applied to more complex tissues such as heart valves, the results have been reported as being unsatisfactory. A study was conducted in which 15 allogeneic heart valves were processed by freeze-drying prior to transplantation. Most of the freeze-dried valves

failed due to mechanical causes in the early post-graft interval. However, those freeze-dried valves which did not fail demonstrated prolonged functionality (up to 15 years).

The use of enzymes and detergents in the processing of tissues have also been used to remove antigenic cells from collagen-based transplantable tissues. Organic solvents and detergent treatments have been used successfully with relatively simple tissues such as dura matter used in reconstructive surgical procedures. Chemical processing of more complex structures such as heart valves, vascular grafts and skin, however, has had only limited success in clinical applications.

SUMMARY OF THE INVENTION

The present invention is directed to a method of processing and preserving collagen based tissues for use as transplantable tissues. The present invention is also directed to the product of the method of processing.

Generally the process of preserving collagen-based tissues of the present invention includes: procuring the collagen-based tissue; treating the tissue in a first detergent solution; treating the tissue in an enzyme solution; treating the tissue in a cryopreservation solution; and, cryopreserving the tissue. The process also includes treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the Maillard reaction and the subsequent formation of advanced glycosylation end products; treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via reactive oxidative species of molecules; and treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the formation and propagation of molecular free radicals. The process may be applied to a collagen based tissue that is a heart valve or vascular conduits such as veins or arteries, nerve or nerve tissues, umbilical cord vessels, dura, dermis and the like. The first detergent solution may be formulated so as to include one or more of t-octylphenoxypolyethoxyethanol (Triton X-100), n-octyl- ß-D-glucopyranoside, deoxycholate, octanoic acid (caprylate), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), ethylene diamine tetraacetate (EDTA), sodium chloride, and broad-spectrum antimicrobials in a physiological buffer solution. A second detergent solution may alternatively be included in the process and such second detergent solution may be formulated from the same ingredients as the first

detergent solution. The enzyme solution may include one or more of DNase Type I, DNase Type II, RNase, phospholipase A, phospholipase C, and broad-spectrum antibiotics in a physiological buffer solution including broad spectrum antimicrobials.

During the course of treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the Maillard reaction and the subsequent formation of advanced glycosylation end products, the techniques and solution utilized may be selected from: depressed temperatures; use of non-reactive or non-reducing carbohydrates such as those that result from the molecular reduction of the carbonyl group of glucose and related compounds, and aminoguanidine; and combinations thereof.

During the process in which the treating of the tissue takes place so as to prevent or inhibit the molecular crosslinking of processed tissues via reactive oxidative species of molecules, the techniques used may include: depressed temperatures; inert atmosphere; and one or more of deferoxamine mesylate, dimethyl sulfoxide (DMSO), catalase, superoxide dismutase, a-tocopherol, reduced glutathione, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; and combinations thereof. The step of treating the tissue so as to prevent or inhibit propagation of molecular free radicals includes: using depressed temperatures; and one or more of dimethyl sulfoxide (DMSO), a-tocopherol, ascorbate, reduced glutathione, flavonoids, and inositol hexaphosphoric acid (phytic acid). The cryopreservation solution utilized during cryopreservation may include non-reducing maltodextrin and ethylene diamine tetraacetate (EDTA) and a physiologic buffer solution.

These and other features of the present invention are more fully set forth in the following description of illustrative embodiments of the invention.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention is generally directed to a method of processing and preserving collagen based tissues, and in particular cardiovascular tissues and nerve tissues, for use as transplantable tissues. Cardiovascular tissues included, but are not limited to, heart valves, arteries, veins and umbilical vessels. Nerve tissue may include, central nervous tissues such as dura, as well as nerves and other collagen based tissues of the central and peripheral nervous systems. The present invention is also directed to the product of the method of processing.

As disclosed herein, the process of the present invention generally includes procuring donor tissue from a donor source. Treating the donor tissue in a detergent solution, and treating in an enzyme solution so as to simultaneously remove harmful elements and give an acellular collagen matrix. The process may include the use of several different detergent or enzyme solutions or alternatively the method may utilize a combination of detergent and enzyme solutions. The process further includes protecting the matrix against non-enzymatic crosslinking due to the Maillard reaction or its subsequent end products, reactive oxidative molecular species, or molecular free radicals. This can be achieved by introducing non-participants and/or inhibitors to these reactions. Preservation of the resulting acellular collagen matrix is carried out by cryopreservation, preferably by molecular distillation drying, to give a dried and hence preserved, acellular collagen matrix. The resulting preserved, acellular collagen matrix may be rehydrated and used as a transplant alleviating the need for a conventional autologous, allogeneic tissues or a man-made synthetic transplant.

Procurement of Donor Tissue: Upon consideration of the disclosure contained herein, one of ordinary skill in the art should understand that a wide range of collagen based tissues may be processed using the principles of the present invention. That is to say, the processing and preserving of collagen based tissues such as cartilaginous tissues, such as tendons, ligaments, sinus tissue and the like; dura matter of the central nervous system, spinal cord, peripheral nerve, and other similar neurological tissues, dermis and other skin tissue, vascular tissues such as veins and arteries heart valves and the like, umbilical cord vessels, corneal tissue and other collagen containing tissue of the ocular system, periodontal tissues such as the gingiva and other such soft tissues that contain a collagen matrix are considered to be within the scope of the present invention. One of skill in the art should understand that depending on the size, shape, cellular structure and condition of the collagen based tissue upon harvest, a variety of surgical techniques may be needed to obtain the donor tissue samples. Such techniques should be known to skilled surgeons, but should also be appreciated by one of ordinary skill in the art.

A suitable donor source must be located for the procurement of the donor tissue. This donor source may be either a human cadaver or an appropriately sized animal. Generally the donor should be in such condition that the tissues of interest have not undergone irreversible,

adverse biochemical or mechanical changes. In one preferred embodiment, the donor is a freshly killed pig which has not undergone scalding. In another preferred embodiment, the donor is a human donor who has indicated the desire to be a tissue donor upon death. The donor tissue is dissected and removed from the donor. In addition to the tissue, a sample of the surrounding tissue should be taken for cryosection and testing. Typically the sample of surrounding tissue is frozen immediately after procurement. The donor tissue, should be procured such that there is sufficient geometry for transplantation.

The donor tissue may be placed into a stabilizing solution in circumstances in which the process of the present invention is not to be carried out soon after procurement. Stabilizing solution may include cold (i.e. 4 °C) Roswell Park Memorial Institute (RPMI) solution or University of Wisconsin (UW) solution with additional antibiotics. Suitable antimircobial suchas cefoxitin, lincomycin, polymyxin, vancomycin and amphotericin. The tissue in cold (i.e. 4 °C) stabilizing solution is shipped as soon as possible for processing as disclosed herein. Upon receipt, a second sample of the tissue is taken to determine its suitability for further processing.

Decellularization: The donor tissue prior to treatment is rinsed in a physiological saline solution to remove excess blood and tissue particles that may be present. The donor tissue is then immersed in a first detergent solution. The general purpose of this first detergent solution is to remove, as completely as possible, any residual blood elements which might contribute oxidative or enzymatic compounds which could damage the tissue. The first detergent solution is formulated to contain a detergent and sodium chloride for solubilizing lipid membranes and proteins, and a divalent cation chelator to inhibit protease activity, in buffered water or culture medium. In one embodiment the first detergent solution is comprised of 0.24 mM t-octylphenoxypolyethoxyethanol (Triton X-100), 25 mM ethylene diamine tetraacetate (EDTA), and 1M sodium chloride in a 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer. Alternatively, the first detergent solutions may be formulated to include TX-100, sodium chloride, and EDTA in a Roswell Park Memorial Institute (RPMI) solution. Sufficient first detergent solution should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid

surrounding the tissue. Upon completion of the immersion step, the first detergent solution is drained from the donor tissue, with the solution being discarded.

After sufficient time has elapsed to remove all the residual blood elements the tissue is transferred to a vitrification solution maltodextrin (VSMD). VSMD is a solution that, in this instance, promotes solubilization of cellular proteins by enhancing detergent entry into the tissue via osmotic changes, and by providing carbohydrates which directly enhance the solubility of specific cellular proteins. The components of the VSMD solution should include polymers of polyhydroxy compounds which may be of low browning (Maillard reaction) potential, chelators of divalent cations to inhibit proteolytic activity, water, and a suitable buffer for maintaining the desired pH. The maltodextrin may be chemically modified to a dextrose equivalence (DE) less than 1 in order to prevent its participation in the Maillard reaction. This reaction, if unchecked, can result in non-enzymatic crosslinking of the matrix. In a preferred embodiment, VSMD includes modified 75% maltodextrin, about 10 mM ethylene diamine tetraacetate (EDTA), water, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). The pH of the VSMD solution is preferably adjusted to about pH 7.4 to about pH 7.5. Sufficient VSMD should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. Upon completion of the immersion step, the VSMD is drained from the donor tissue, with the solution being discarded.

After completion of draining, the donor tissue is placed in a second detergent solution and gently agitated. The second detergent solution is a solution that that disrupts and solubilizes cellular membranes and potentially antigenic components so that they may be washed free of the tissue. The components of the second detergent solution include detergents and divalent cation chelators in buffered water or cell culture medium, plus broad range antimicrobials, antifungals, anti-oxidants and free radical scavengers. In one particular embodiment, the second detergent solution includes n-octyl- ß-D-glucopyranoside, ethylene diamine tetraacetate (EDTA), deferoxamine, phytic acid, and aminoguanidine in degassed Roswell Park Memorial Institute (RPMI) solution. Roswell Park Memorial Institute (RPMI) solution is a commercially available cell culture medium. In another embodiment, the second detergent solution includes about 40

mM n-octyl-ß-D-glucopyranoside, about 25 mM ethylene diamine tetraacetate (EDTA), about 10 mM deferoxamine, about 10 mM phytic acid, and about 100 mM aminoguanidine in degassed Roswell Park Memorial Institute (RPMI) solution at a pH of about 7.4 to 7.5. In addition to the above components, the second detergent solution may contain antimicrobials to prevent transmission of bacterial or fungal infection. Suitable antimicrobials include one or a combination of penicillins, aminoglycosides, cephalosporins, erythromycins, tetracyclines, polymyxins, antifungals, or others with suitable activity against expected organisms in the tissue procurement setting. In one particular example, antimicrobials include lincomycin, polymyxin B sulfate, cefoxitin, vancomycin, and amphotericin B. In another example, the second detergent solution includes 250 mM lincomycin, 1000 activity units/ml polymyin B sulfate, 0.5 M cefoxitin, 20 mM vancomycin, and 25 mM amphotericin B. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. The duration and temperature of optimal treatment in the second detergent solution may vary with differing types of tissue, however, one skilled in the art should know how to systematically vary each parameter to obtain the optimum conditions.

The tissue may be trimmed to final specifications upon completion of the second detergent treatment. The resulting treated tissue should be washed with sterile physiological salt solution, preferably at a pH the same as the detergent, and more preferably at a pH from about 7.4 to about 7.5.

The washed treated tissue is placed in an enzyme solution and is incubated for a suitable temperature and time. The purpose of the enzyme solution is to disrupt and solubilize specific cellular components, such as the cell nucleus and cell phospholipids. The enzyme solution of the present invention includes DNase I, deferoxamine, phytic acid, and aminoguanidine in a 20 mM HEPES buffered water. The enzyme solution may also include phospholipase C to assist the clearance of phospholids. Other agents with activity against non-enzymatic crosslinking of the tissue may also be included in the formulation of the enzyme solution, such as dimethyl sulfoxide (DMSO), catalase, superoxide dismutase, a-tocopherol, reduced glutathione, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ascorbate, flavonoids, and inositol hexaphosphoric acid (phytic acid). In addition, antimicrobials such as one or a combination of

penicillins, aminoglycosides, cephalosporins, erythromycins, tetracyclines, polymyxins, antifungals, or others with suitable activity against expected organisms in the tissue procurement setting may be added to the enzyme solution. In one preferred embodiment, the enzyme solution includes 150 activity units/mg DNase I, 10 mM deferoxamine, 100 mM phytic acid, and 100 mM aminoguanidine. In a more preferred embodiment, the enzyme solution also includes 250 mM lincomycin, 1000 activity units/ml polymyin B sulfate, 0.5 M cefoxitin, 20 mM vancomycin, and 25 mM amphotericin B as antimicrobials. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. The donor tissue is preferably incubated in the enzyme solution at 37°C. Depressed temperatures may be used as well, however, to decrease the rate of reaction for non-enzymatic crosslinking within the tissue. After completion of the treatment with the enzyme-containing solution, the enzyme solution is decanted and discarded. The tissue is washed three times with sterile degassed, physiological salt solution using fresh solution for each wash.

Cryopreservation: When cell viability or tissue ultrastructure are to be preserved following cooling, two methods are generally available. The first is to ultrarapidly cool the sample, resulting in the tissue fluids being vitrified, i.e., frozen in the absence of ice crystals. The second is to incorporate chemical additives to confer a degree of cryoprotection and allow vitification at slower cooling rates.. The chemicals range from naturally occurring cryoprotectants such as glycerol, proline, sugars, and alcohols to organic solvents such as dimethylsulfoxide (DMSO) to high molecular weight polymers such as polyvinylpyrrolidone (PVP), dextrin, maltodextrin and hydroxyethyl starch (HES).

Vitrification of cells and tissues is limited by the rate at which the sample can be cooled and the insulating properties of the tissue itself. Due to physical limitations, one can only achieve vitrification of a thin layer of tissues using state of the art techniques. Thus chemical agents for cryoprotection and manipulating the cooling rate are utilized to cool and store biological samples without causing structural and functional damage.

The cooling rate in the presence of cryoprotective compounds is a significant factor in freezing injury. Normally for cells, slow cooling is better than elevated cooling rates since the latter promotes intracellular ice formation. This occurs because there is insufficient time for

water to escape from the cells before the contained cell water freezes. With slow rate cooling, extracellular ice forms first, resulting in dehydration of the cell which, together with the presence of the cryoprotectant, prevents intracellular ice formation. For tissue matrix samples there is a more direct correlation to the overall reduction in the degree of total ice crystal formation.

A source of damage to frozen tissue, other than freezing itself, is the osmotic and toxic effects of many of the cryoprotective agents. The physicochemical effects of cryoprotectants are (a) depression of the equilibrium freezing point of substrate and cytoplasm on a colligative basis, (b) depression of homogeneous ice nucleation temperature, (c) reduced rate of ice crystal growth due to change in the viscosity and thermal diffusivity of the solution, and (d) dehydrative effects on cells by osmotic action. When used in mixtures, some cryoprotective compounds may counteract the toxicity of other cryoprotectants. The factors affecting the cryoprotective nature of compounds are (a) chemical nature, (b) relative lack of toxicity, (c) molecular size and penetrating ability, and (d) interaction with other compounds in the mixture.

Upon completion of the procurement and decellularization steps, the tissue is incubated in a second vitrification solution maltodextrin (VSMD) for a second time. The second VSMD may have the same formulation as previously disclosed above. In one preferred embodiment the second VSMD is formulated to comprise a 75% maltodextrin and the other previously disclosed components. However, if the formation of some ice crystals can be tolerated, the second VSMD may utilize lower amounts of maltodextrin preferably about 35% maltodextrin may be used. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. After a sufficient incubations time in the second VSMD, preferably four hours or more, the donor tissue is removed from the solution and incubated in fresh second VSMD for an additional minimum of four hours. After completion of infiltration with the second VSMD , the tissue is drained and placed in TYVEK® pouches for cooling and drying.

Cooling Parameters: With the tissue sealed in TYVEK® pouches, it is cryofrozen in a manner which prevents or limits the amount of ice crystal formation over the course of the drying process.

For purposes of cryopreparation of the biological suspensions of this invention, it is essential to note that a variety of cooling processes can be used. In a preferred embodiment of

this invention, rapid cooling is considered essential to obtain the proper ice crystal blend. In the most preferred embodiment of this invention, a vitrification procedure is used which results in the formation of a substantial proportion of amorphous water in the biological sample. Regardless of the form of cooling that is used, it is believed that amorphous phase water, cubic ice crystals and hexagonal ice crystals are present in the final product. The method of cooling has a distinct bearing on the distribution of ice crystal types found in the cooled cryosolution. In a preferred embodiment, samples are cooled by an appropriate method such that ice crystal formation is below the degree that would cause damage to the sample. Once frozen, the sample is then stored below the transition temperature of the most unstable ice form. For amorphous ice, this is preferentially below -160°C.

Drying Parameters: The aim of controlled drying of a frozen biological sample by molecular distillation drying is to remove water from the sample without further mechanical or chemical damage occurring during the drying process. This involves avoiding, by use of appropriate drying conditions, two fundamental damaging events. The first is to remove water from ice crystalline phases without transition to larger more stable and more destructive crystals. The second is to remove water from solid but noncrystalline water or water-solute mixtures without melting or crystallization of these solid phases. This second component refers to water present in the amorphous condition, water together with solute in the eutectic or water together with a compound which binds and structures water and hence, prevents its crystallization during the freezing process. Hence, vitreous water can be of low energy and stability, as in ultrarapidly-cooled pure water, or high energy and stability, as that achieved with cryoprotective agents with intermediate rates of cooling.

Many of the features required of controlled drying to avoid the occurrence of these events are overlapping. The reason for this is that each form of water will have a particular energy state, whether in a crystal or bound to a cryoprotective compound, and it is this energy state, rather than its configuration, which determines the requirements for drying. The aim of controlled drying is to remove water from the cubic ice phase during its transition to a hexagonal phase and in a time less than is required for any significant transition to hexagonal ice phase to occur.

This argument can be applied repetitively to all forms of water present whether it be crystalline in the form of cubic or hexagonal or noncrystalline as amorphous or bound to any molecule, be it cryoprotectant, protein, carbohydrate, or lipid. To simplify this concept, water in a frozen biological sample can be described as having a specific energy level E. In a frozen biological sample, there will be water forms of multiple definable energy levels:

$$E_1 \quad E_2 \quad E_3 \quad \text{----} \quad E_n$$

The mode of preparation, the nature of the sample, the use of cryoprotectants or other additives, and the cooling rate used will determine the relative proportions of these different water forms. Each energy level will determine the onset temperature of its transition or melting and the temperature dependence of the rate of the transition or melt.

The controlled drying processes must be able to remove each of these different states of water during the transition from one phase to another second phase and in less time than is required to complete the transition. This mode of drying, therefore, requires that several conditions be met.

First, the sample should be loaded into the dryer without temperature elevation above its lowest transition temperature. If elevation of temperature does occur, this should be over a short period of time such that no appreciable transition occurs. Ideally, loading occurs under liquid nitrogen at -190°C, well below the lowest discernible transition of -160°C for pure, ultrarapidly-cooled amorphous water. If, however, the sample is predominantly cubic ice or a mixture of water and cryoprotectants with a glass transition of the order of -100°C to -130°C, a closed circuit refrigeration system may be sufficient to enable maintenance of the sample temperature below the onset of transition.

Second, once loaded, the sample should be exposed to vacuum and be in direct line of sight of the condenser surfaces. The criteria for these are again determined by the nature of the water phases present in the sample.

Third, the vacuum within the chamber during the drying of a particular phase should create a partial pressure of water at least equivalent to or less than the saturation vapor pressure of water in the phase to be removed. This saturation vapor pressure is dependent on the nature of the water phase and its temperature. Hence, for pure amorphous water in the transition range

of -160°C to -130°C, the approximate saturation vapor pressures are 6×10^{-12} mbar (-160°C) and 5×10^{-7} mbar (-130°C), respectively. As the transition times of amorphous to cubic ice in this same temperature range, -160°C to -130°C, vary from 5×10^{5} minutes to 5 minutes, drying will be very slow until temperatures of the order of -150°C to -140°C are reached requiring a vacuum of 5×10^{-10} to 2×10^{-8} mbar. For cubic ice, little if any drying will occur below its onset of transition at -130°C as its saturation vapor pressure will be of the order of one log lower than for amorphous water. In the transition range, -130°C to -100°C, the saturation vapor pressure of cubic ice is approximately 5×10^{-8} to 9×10^{-5} mbar. The transition times of cubic to hexagonal are 700 minutes and 109 minutes respectively.

The saturation vapor pressure, therefore, determines the vacuum requirements for drying and can be applied to all water phases present. It is important to note that the same vacuum criteria are not applicable to all phases, but rather are phase-dependent.

Another criteria of the vacuum is that the mean free path created be in excess of the distance between the sample and the condenser surface. Ideally, this should be a tenfold excess. The condenser surface should be a lower temperature than the onset transition temperature of the phase of water being removed from the sample so that the saturation vapor pressure of water condensed on this surface during drying is considerably lower than that of the water phase within the sample. Ideally, this should be three orders of magnitude lower. For a sample containing multiple water phases, the temperature of the condenser surface should remain below the onset of transition of the least stable ice phase remaining to be removed. Ideally, the condenser should also be in line of sight of the sample.

Once the sample has been loaded and exposed to vacuum and the condenser surfaces, the sample and sample holder should be heated so as to increase the mobility of water molecules and hence, cause their escape. This is an important component in the drying of a sample containing multiple phases or energy levels of water. The temperature of the sample should be accurately known and the control of temperature and the rate of sample heating should be accurately controlled. This is to ensure that the drying of each phase of water in the sample is sequential.

Hence, for a sample containing multiple phases of water of energy level E_1, and E_2 ---- E_n where E_1 is the least stable, then heating must occur at such a rate that E_1 is removed prior to

its transition to E_2. E_2 prior to its transition to E_3 and so on. This requires nonequilibrium drying conditions and heating at a continuous rate or by holding at a constant temperature level such that sublimation occurs as determined by:

$$Js = NPs\left(\frac{M}{2\pi QT}\right)^{0.5}$$

where Js = sublimation rate in g cm^{-1} sec^{-1}

N = coefficient of evaporation

Ps = saturation vapor pressure

M = molecular weight of water

Q = universal gas constant

T = absolute temperature of the sample.

This is consistent with the transition rate for the particular phase being removed. For example, the rate of the amorphous to cubic transition is given by:

$$E = 2.04 \times 10^{28} \times \exp(-0.465T)$$

Alternatively, if the transition window is T_1 to T_2, the sublimation rate and the transition rate will vary with temperature during this interval. The rate of heating during this window T_1 to T_2 must be such that sublimation occurs throughout the dimensions of the sample before transition at any particular temperature is completed.

In this way, the aim of controlled molecular distillation drying is achieved, i.e., the sequential removal of each phase of water under conditions appropriate to the properties of each phase without appreciable ice crystal growth, formation or melting of the particular phase.

In a preferred embodiment, samples are cooled by an appropriate method such that ice crystal formation is below the degree that would cause damage to the sample. This may be by immersion in liquid nitrogen, exposure to liquid nitrogen vapor, or other similar rapid freezing

process. Once frozen, the sample is then stored below the transition temperature of the most unstable ice form. For amorphous ice, this is preferentially below -160°C. The sample is then loaded into a sample holder, precooled to -196°C and transferred into a molecular distillation dryer. The dryer chamber is then closed and sealed for vacuum integrity. To avoid recrystallization, the hydrated sample must remain below the transition temperature of the most unstable ice form throughout all manipulations.

Once the sample is loaded, high vacuum (10^{-8} to 10^{-6} mbar) is generated inside the chamber. The sample is placed considerably closer to the condenser surface (liquid nitrogen cooled chamber walls) than the mean free path within the chamber. The condenser temperature should be below that of the sample. For an amorphous sample, the condenser is preferentially -196°C.

The sample holder is then heated via a programmable heater microprocessor thermocouple loop. Heating programs are determined according to the nature of the sample. A typical program for a sample containing amorphous, cubic and hexagonal ice is 10°C per hour from -180°C to -150°C, 1°C per hour from -150°C to -70°C, and 10°C per hour from -70°C to +20°C.

Once dry, the sample must be physically or mechanically isolated from water on the condenser surface or any other source and stored in a closed container either under vacuum or dry inert gas. The sample can be sealed inside an appropriate container within the vacuum chamber and unloaded for subsequent storage and packaging. In one configuration, the sample is contained within a glass vial and sealed with a butyl rubber lyophilization stopper at the end of cycle. More specific details of the operation of the molecular distillation dryer and the above process of drying is described in U.S. Patent No. 4,865,871. 5,336,616, the entire contents of which are hereby incorporated herein by reference.

Upon consideration of the disclosure contained herein, one of ordinary skill in the art should understand that a wide range of collagen-based tissues may be processed using the principles of the present invention. That is to say, the processing and preserving of collagen based tissues, such as cartilaginous tissues such as tendons, ligaments, sinus tissue and the like; dura mater of the brain, spinal cord, peripheral nerve and other similar neurological tissues,

dermis and other skin tissue, vascular tissues such as veins and arteries, heart valves and the like, corneal tissue and other collagen containing tissue of the ocular system, periodontal tissues such as the gingiva and other such soft tissues that contain a collagen matrix are considered to be within the scope of the present invention. One of skill in the art should understand that depending on the size, shape, cellular structure and condition of the collagen based tissue upon harvest, the specific conditions, such as concentration, time, temperature and the like may be required. Such adjustments may be determined by routine variation of parameters disclosed herein and thus may be used to substantially achieve the results of the present invention.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the scope of the invention.

Example 1.

Procurement: The donor tissue is provided by a freshly killed pig which has not undergone scalding. The heart valve should be dissected from the donor in a manner such that there is sufficient coronary and muscle skirt lengths to facilitate subsequent surgical implantation. If necessary the donor tissue may be placed in a stabilization fluid for transportation. However, in the present case this was not necessary.

Decellularization: The trimmed tissue is rinsed in a physiological saline solution to remove excess blood and tissue particles. It is then placed in the first detergent solution for about 1 hour at about 4 °C The first detergent solution is comprised of about 0.24 mM t-octylphenoxypolyethoxyethanol (Triton X-100), about 25 mM ethylene diamine tetraacetate (EDTA), and about 1M sodium chloride in an about 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer. Alternatively, TX-100, sodium chloride, and EDTA in Roswell Park Memorial Institute (RPMI) solution may be used as the first detergent solution. Sufficient first detergent solution should be used so that the donor tissue is completely immersed

in the solution. Agitation should be provided so that all surfaces are well washed. Upon completion of the immersion step, the first detergent solution is drained from the donor tissue, with the first detergent solution being discarded.

After about one hour the tissue is transferred to vitrification solution maltodextrin (VSMD) and the tissue is incubated in VSMD for about 1 to 4 hours. The VSMD includes modified 75% maltodextrin, about 10 mM ethylene diamine tetraacetate (EDTA), water, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). The pH of the VSMD solution is preferably adjusted to about pH 7.4 to pH 7.5. Sufficient VSMD should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. Upon completion of the immersion step, the VSMD is drained from the donor tissue, with the solution being discarded.

After completion of draining, the donor tissue is placed in a second detergent solution and gently agitated. For the present example, heart valves are agitated in the second detergent solution at room temperature for about 24 hours. The second detergent solution includes about 40 mM n-octyl-ß-D-glucopyranoside, about 25 mM ethylene diamine tetraacetate (EDTA), about 10 mM deferoxamine, about 10 mM phytic acid, and about 100 mM aminoguanidine in degassed Roswell Park Memorial Institute (RPMI) solution at a pH of about 7.4 to 7.5. The second detergent solution may optionally include about 250 mM lincomycin, about 1000 activity units/ml polymyin B sulfate, about 0.5 M cefoxitin, about 20 mM vancomycin, and about 25 mM amphotericin B.

The resulting treated tissue is washed–with sterile physiological salt solution, preferably at a pH the same as the detergent, and more preferably at a pH from about 7.4 to about 7.5.

The washed treated tissue is placed in an enzyme solution that includes about 150 activity units/mg DNase Type I, about 10 mM deferoxamine, about 100 mM phytic acid, and about 100 mM aminoguanidine in a 20 mM HEPES buffered water. In a more preferred embodiment, the DNase solution also includes about 250 mM lincomycin, about 1000 activity units/ml polymyin B sulfate, 0.5 M cefoxitin, about 20 mM vancomycin, and about 25 mM amphotericin B as antimicrobials. The heart valves are incubated in the enzyme solution for about 24 hours at about 37°C. Sufficient enzyme solution should be used so that the donor tissue

is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. After completion of the treatment with the enzyme-containing solution, the enzyme solution is decanted and discarded. The tissue is washed three times with sterile degassed, physiological salt solution using fresh solution for each wash.

Cryopreservation: Upon completion of the above steps, the tissue is incubated in a second VSMD for at least four hours. The second VSMD is formulated so as to have the same composition and the first VSMD. Sufficient VSMD should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. After incubation the tissue is removed from the solution and incubated in fresh VSMD solution for an additional minimum of four hours. After completion of this second VSMD infiltration, the tissue is drained and placed in TYVEK® pouches.

With the tissue sealed in TYVEK® pouches, it is cryofrozen and freeze-dried in a manner which prevents or limits the amount of ice crystal formation as described above. The entire drying process takes place over the course of about three days. Typically this process involves cooling the sample below about -160 °C, placing the cooled sample under vacuum and warming as described above. A programmable temperature controller can be used during this process to ensure consistency and accuracy. Upon reaching room temperature the dried tissue sample is transferred and packaged to prevent contact with ambient moisture and under sterile conditions.

Example 2.

Procurement: The donor tissue is provided by a human donor. A radial artery, saphenous vein or umbilical vessel should be dissected from the donor in a manner such that there is sufficient tissue to facilitate subsequent surgical implantation. The donor tissue is placed in a stabilization fluid for transportation. Stabilizing solution may include cold Roswell Park Memorial Institute (RPMI) solution or University of Wisconsin (UW) solution with additional antibiotics. Suitable antimicrobials include:cefoxitin, lincomycin, polymyxin, vancomycin, and amphotericin. The tissue in cold stabilizing solution is shipped as soon as possible for

processing. Upon receipt at the processing facility, a sample is taken of the tissue to determine its suitability for further processing.

Decellularization: The trimmed tissue is rinsed in a physiological saline solution to remove excess blood and tissue particles. It is then placed in the first detergent solution for about 1 hour at about 4 °C. The first detergent solution is comprised of about 0.24 mM t-octylphenoxypolyethoxyethanol (Triton X-100), about 25 mM ethylene diamine tetraacetate (EDTA), and about 1M sodium chloride in a about 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer. Alternatively, Triton X-100, sodium chloride, and EDTA in Roswell Park Memorial Institute (RPMI) solution may be used as the first detergent solution. Sufficient first detergent solution should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so that all surfaces are well washed. Upon completion of the immersion step, the first detergent solution is drained from the donor tissue, with the solution being discarded.

After about one hour the tissue is transferred to vitrification solution maltodextrin (VSMD) and the tissue is incubated in VSMD for approximately 1 to 4 hours. The VSMD includes modified 75% maltodextrin, about 10 mM ethylene diamine tetraacetate (EDTA), water, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). The pH of the VSMD solution is preferably adjusted to about pH 7.4 to pH 7.5. Sufficient VSMD should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. Upon completion of the immersion step, the VSMD is drained from the donor tissue, with the solution being discarded.

After completion of draining, the donor tissue is placed in a second detergent solution and gently agitated. For the present example, heart valves are agitated in the second detergent solution at room temperature for about 24 hours. The second detergent solution includes about 40 mM n-octyl-ß-D-glucopyranoside, about 25 mM ethylene diamine tetraacetate (EDTA), about 10 mM deferoxamine, about 10 mM phytic acid, and about 100 mM aminoguanidine in degassed Roswell Park Memorial Institute (RPMI) solution at a pH of about 7.4 to 7.5. The second detergent solution may optionally include about 250 mM lincomycin, about 1000 activity

units/ml polymyin B sulfate, about 0.5 M cefoxitin, about 20 mM vancomycin, and about 25 mM amphotericin B.

The resulting treated tissue is washed–with sterile physiological salt solution, preferably at a pH the same as the detergent, and more preferably at a pH from about 7.4 to about 7.5.

The washed treated tissue is placed in an enzyme solution that includes about 150 activity units/mg DNase Type I, about 10 mM deferoxamine, about 100 mM phytic acid, and about 100 mM aminoguanidine in a 20 mM HEPES buffered water. In a more preferred embodiment, the DNase solution also includes about 250 mM lincomycin, about 1000 activity units/ml polymyin B sulfate, 0.5 M cefoxitin, about 20 mM vancomycin, and about 25 mM amphotericin B as antimicrobials. The heart valves are incubated in the enzyme solution for about 24 hours at about 37 °C. Sufficient enzyme solution should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. After completion of the treatment with the enzyme-containing solution, the enzyme solution is decanted and discarded. The tissue is washed three times with sterile degassed, physiological salt solution using fresh solution for each wash.

Cryopreservation: Upon completion of the above steps, the tissue is incubated in a second VSMD for at least four hours. The second VSMD is formulated so as to have the same composition and the first VSMD. Sufficient VSMD should be used so that the donor tissue is completely immersed in the solution. Agitation should be provided so as to promote the mixing and flow of the fluid surrounding the tissue. After incubation the tissue is removed from the solution and incubated in fresh VSMD solution for an additional minimum of four hours. After completion of this second VSMD infiltration, the tissue is drained and placed in TYVEK® pouches.

With the tissue sealed in TYVEK® pouches, it is cryofrozen and freeze-dried in a manner which prevents or limits the amount of ice crystal formation as described above. The entire drying process takes place over the course of about three days. Typically this process involves cooling the sample below about -160 °C, placing the cooled sample under vacuum and warming as described above. A programmable temperature controller can be used during this

process to ensure consistency and accuracy. Upon reaching room temperature the dried tissue sample is transferred and packaged to prevent contact with ambient moisture and under sterile conditions.

Example 3. (Nerve)

Procurement:

Nerve tissue is procured and immediately placed into sterile Roswell Park Memorial Institute (RPMI) solution with or without antimicrobials including 240 mg/l cefoxitin, 120 mg/l lincomycin, 100 mg/l polymyxin B, 30 mg/l vancomycin and 25 mg/lamphotericin. The tissue may remain in this solution for up to about 7 days provided the solution is changed approximately every 48 hours with fresh sterile solution. Ideally the processing of this tissue would begin within about 24 hours of procurement.

Decellularization: The transport solution (RPMI) is removed and replaced with the first decellularizing solution consisting of a sodium phosphate buffer (about 0.639% Na_2HPO_4 and about 0.069 % NaH_2PO_4) plus about 0.5 % Triton X-100 (n-octyl-ß-D-glucopyranoside), about 1 M NaCl, about 10 mM EDTA. This solution may optionally include antimicrobials such as cefoxitin, lincomycin, polymyxin, vancomycin, ans amphotericin. The tissue is rinsed in this solution for about 5-30 minutes at room temperature with gentle agitation. The solution is replaced with fresh solution and agitated for an additional about 15-24 hours.

The tissue is removed from this solution and washed two times for about 15 minutes each in phosphate buffered saline containing about 10 mM EDTA. The tissue is then rinsed for about 5-30 minutes followed by approximately a 15-24 hour incubation at 37°C in a second decellularizing solution consisting of about 5.06 % n-octanoic acid (Caprylic acid) in a sodium phosphate buffer consisting of about 0.639% Na_2HPO_4 and about 0.069 % NaH_2PO_4 with or without antibiotics. Alternatively this second decellularizing solution may consist of about 2 % deoxycholate in about 10 mM HEPES buffer with or without antibiotics.

The tissue is removed from this solution and washed two times for about 15 minutes each in phosphate buffered saline containing about 10 mM EDTA. The tissue is then incubated for about 15-24 hours at room temperature with agitation in a solution consisting of about 50 ug/ml Dnase-I in about 0.9 % NaCl and about 10 mM $MgCl_2$ at a pH of about 7.4. The tissue is

removed from this solution and washed three times for about 15 minutes each in phosphate buffered saline containing about 10 mM EDTA.

Cryopreservation: The tissue is then incubated for about 1-6 hours at room temperature with agitation in a solution consisting of about 35 % maltodextrin and about 10 mM EDTA in about 10 mM HEPES buffer. The tissue is then sealed in TYVEK pouches, cryofrozen and freeze-dried in a manner which prevents or limits the amount of ice crystal formation. The entire drying process takes place over the course of about three days. Typically this process involves cooling the sample below about -160 °C, placing the cooled sample under vacuum and warming as described above. A programmable temperature controller can be used during this process to ensure consistency and accuracy. Upon reaching room temperature the dried tissue sample is transferred and packaged to prevent contact with ambient moisture and under sterile conditions.

In view of the above one of ordinary skill in the art should appreciate that the present invention includes many possible embodiments One such embodiment includes a process of preserving collagen-based tissues, the process including: procuring the collagen-based tissue; treating the tissue in a detergent solution; treating the tissue in an enzyme solution; treating the tissue via techniques and/or additives which prevent or inhibit the molecular crosslinking of processed tissues via the Maillard reaction and the subsequent formation of advanced glycosylation end products; treating the tissue via techniques and/or additives which prevent or inhibit the molecular crosslinking of processed tissues via reactive oxidative species of molecules; treating the tissue via techniques and/or additives which prevent or inhibit the molecular crosslinking of processed tissues via the formation and propagation of molecular free radicals; treating the tissue in a cryopreservation solution; and, cryopreserving the tissue. In one preferred embodiment the collagen based tissue is a heart valve.

In yet another embodiment the detergent solution includes one or more of t-octylphenoxypolyethoxyethanol (Triton X-100), n-octyl- ß-D-glucopyranoside, deoxycholate, octanoic acid (caprylate), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), ethylene diamine tetraacetate (EDTA), sodium chloride, and broad-spectrum antimicrobials in a physiological buffer solution. In a preferred embodiment the enzyme solution includes one or more of DNase Type I, DNase Type II, RNase, phospholipase A,

phospholipase C, and broad-spectrum antibiotics in a physiological buffer solution including broad spectrum antimicrobials. In one embodiment of the process of the present invention the techniques or inhibitors to molecular crosslinking via the Maillard reaction and the formation of advanced glycosylation end products includes depressed temperatures, use of non-reactive or non-reducing carbohydrates such as those that result from the molecular reduction of the carbonyl group of glucose and related compounds, and aminoguanidine. Another embodiment includes techniques or inhibitors to molecular crosslinking via the reactive oxidative species which include depressed temperatures, inert atmosphere, and one or more of deferoxamine mesylate, dimethyl sulfoxide (DMSO), catalase, superoxide dismutase, a-tocopherol, reduced glutathione, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. In another embodiment the techniques or inhibitors to molecular crosslinking via molecular free radicals includes depressed temperatures and one or more of dimethyl sulfoxide (DMSO), a-tocopherol, ascorbate, reduced glutathione, flavonoids, and inositol hexaphosphoric acid (phytic acid). The cryopreservation solution preferably includes non-reducing maltodextrin and ethylene diamine tetraacetate (EDTA) in a physiologic buffer solution.

Applicants also consider the product of the processes disclosed herein to be part of this invention. Thus in one such embodiment, the product of the process is a preserved collagen based tissue that is suitable for transplantation after it has been rehydrated. In another such embodiment, the product is a preserved heart valve that is suitable for transplantation once it has been rehydrated. Yet another embodiment is sapheous vein or other vascular graft that is suitable for transplantation after it has been rehydrated. Still another embodiment of the present invention is nerve or nervous tissue that is a preserved collagen base tissue that is suitable for transplantation after it has been rehydrated.

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the process described herein without departing from the concept and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope and concept of the invention as it is set out in the following claims.

CLAIMS:

1. A process of preserving collagen-based tissues, the process including:

procuring the collagen-based tissue;

treating the tissue in a detergent solution;

treating the tissue in an enzyme solution;

treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the Maillard reaction and the subsequent formation of advanced glycosylation end products;

treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via reactive oxidative species of molecules;

treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the formation and propagation of molecular free radicals;

treating the tissue in a cryopreservation solution; and,

cryopreserving the tissue.

2. The process of claim 1 wherein the collagen based tissue is selected from heart valve, vein, artery, umbilical vessels, dura, nerve tissue and dermis.

3. The process of claim 2 wherein the detergent solution includes one or more of t-octylphenoxypolyethoxyethanol, n-octyl- ß-D-glucopyranoside, deoxycholate, octanoic acid (caprylate), 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), ethylene diamine tetraacetate (EDTA), sodium chloride, and broad-spectrum antimicrobials in a physiological buffer solution.

4. The process of claim 3 wherein the enzyme solution includes one or more of DNase Type I, DNase Type II, RNase, phospholipase A, phospholipase C, and broad-spectrum antibiotics in a physiological buffer solution including broad spectrum antimicrobials.

5. The process of claim 4 wherein the treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via the Maillard reaction and the subsequent formation of advanced glycosylation end products is selected from: depressed temperatures; use of non-reactive or non-reducing carbohydrates such as those that result from the molecular

reduction of the carbonyl group of glucose and related compounds, and aminoguanidine; and combinations thereof.

6. The process of claim 5 wherein the treating the tissue so as to prevent or inhibit the molecular crosslinking of processed tissues via reactive oxidative species of molecules includes: depressed temperatures; inert atmosphere; and one or more of deferoxamine mesylate, dimethyl sulfoxide (DMSO), catalase, superoxide dismutase, a-tocopherol, reduced glutathione, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; and combinations thereof.

7. The process of claim 6 wherein treating the tissue so as to prevent or inhibit propagation of molecular free radicals includes: depressed temperatures; and one or more of dimethyl sulfoxide (DMSO), a-tocopherol, ascorbate, reduced glutathione, flavonoids, and inositol hexaphosphoric acid (phytic acid).

8. The process of claim 7 wherein the cryopreservation solution includes non-reducing maltodextrin and ethylene diamine tetraacetate (EDTA) and a physiologic buffer solution.

9. The product of the process of claim 8.

10. A process of preserving a collagen based tissues for transplantation, the process comprising:

procuring the collagen based tissue from a donor;

immersing the tissue in a first detergent solution, wherein the first detergent solution includes a detergent and chloride ion for solubilizing lipid membranes and proteins, a divalent cation chelator to inhibit protease activity;

immersing the tissue in a first vitirification solution maltodextrin (VSMD), wherein the first VSMD includes: polymers of polyhydroxy compounds which have a low Maillard reaction potential, a divalent cation chelator and buffer;

immersing the tissue in a second detergent solution, wherein the second detergent solution includes detergent, a divalent cation chelator, an antimicrobial, an antifungal, an antioxidant and a free radical scavenger;

immersing the tissue in an enzyme solution, wherein the enzyme solution includes DNaseI, deferoxamine, phytic acid and aminoguanidine;

immersing the tissue in a second vitrification solution maltodextrine (VSMD) wherein the first VSMD includes: polymers of polyhdroxy compounds which have a low Maillard reaction potential, a divalent cation chelator;

cooling the tissue at a rate and to a temperature such that the formation of ice crystals is substantially prevented;

drying the cooled tissue by molecular distillation drying.

11. The process of claim 10 wherein the collagen based tissue is selected from heart valve, vein, artery, umbilical vessels, dura, nerve tissue and dermis.

12. The product of the process of claim 11.

13. A process of preserving a collagen based tissues for transplantation, the process comprising:

procuring the collagen based tissue from a donor;

immersing the tissue in a first detergent solution, wherein the components of the first detergent solution solubilize lipid membranes and proteins and inhibits protease activity;

immersing the tissue in a first vitirification solution maltodextrin (VSMD), wherein the components of the first VSMD promote solubilization of cellular proteins by enhancing detergent entry into the tissue via osmotic changes and provide carbohydrates to enhance the solubility of the cellular proteins and inhibit proteolytic activity;

immersing the tissue in a second detergent solution, wherein the components of the second detergent solution disrupt and solubilize cellular membranes and antigenic components;

immersing the tissue in an enzyme solution, wherein the components of the enzyme solution disrupt and solubilize the cell nucleus and cell phospholipids and reduce non-enzymatic crosslinking of the tissue;

immersing the tissue in a second vitrification solution maltodextrine (VSMD) wherein the components of the second VSMD promote solubilization of cellular proteins by enhancing detergent entry into the tissue via osmotic changes and provide

carbohydrates to enhance the solubility of the cellular proteins, inhibit proteolytic activity and infiltrates the tissue with a cryoprotectant;

cooling the tissue at a rate and to a temperature such that differing phases of frozen water are formed and the formation of ice crystals is substantially prevented;

drying the cooled tissue by the sequential removal of each phase of frozen water under conditions such that water is removed from the sample without appreciable ice crystal growth, ice crystal formation or melting .

14. The process of claim 13 wherein the collagen based tissue is selected from heart valve, vein, artery, umbilical vessels, dura, nerve tissue and dermis.

15. The product of the process of claim 14.

16. A process of preserving a heart valve for transplantation, the process comprising:

procuring the heart valve from a donor;

immersing the heart valve in a first detergent solution, wherein the first detergent solution includes a t-octylphenoxypolyethoxyethanol and sodium chloride for solubilizing lipid membranes and proteins, ethylenediamine tetraacetate (EDTA) and a buffer;

immersing the heart valve in a first vitirification solution maltodextrin (VSMD), wherein the first VSMD includes: maltodextrin having a low Maillard reaction potential, EDTA and a buffer;

immersing the heart valve in a second detergent solution, wherein the second detergent solution includes n-octyl-ß-D-glucopyranoside, EDTA, deferoxamine, phytic acid, and aminoguanadine in degassed cell culture media;

immersing the heart valve in an enzyme solution, wherein the enzyme solution includes DNaseI, deferoxamine, phytic acid, aminoguanidine and a buffer;

immersing the heart valve in a second vitrification solution maltodextrine (VSMD) wherein the first VSMD includes: maltodextrin having a low Maillard reaction potential, EDTA and a buffer;

cooling the heart valve at a rate and to a temperature such that the formation of ice crystals is substantially prevented so as to give a frozen heart valve;

drying the frozen heart valve by molecular distillation drying.

17. A process of preserving a vascular tissue for transplantation, the process comprising:

procuring the vascular tissue from a donor;

immersing the vascular tissue in a stabilization solution, wherein the stabilization fluid includes antibiotics and tissue culture media;

immersing the vascular tissue in a first detergent solution, wherein the first detergent solution includes a t-octylphenoxypolyethoxyethanol, sodium chloride, ethylenediamine tetraacetate (EDTA) and a buffer;

immersing the vascular tissue in a first vitirification solution maltodextrin (VSMD), wherein the first VSMD includes: maltodextrin having a low Maillard reaction potential, EDTA and a buffer;

immersing the vascular tissue in a second detergent solution, wherein the second detergent solution includes n-octyl-ß-D-glucopyranoside, EDTA, deferoxamine, phytic acid, and aminoguanadine in degassed cell culture media;

immersing the vascular tissue in an enzyme solution, wherein the enzyme solution includes DNaseI, deferoxamine, phytic acid, aminoguanidine and a buffer;

incubating the vascular tissue in a second vitrification solution maltodextrine (VSMD) wherein the first VSMD includes: maltodextrin having a low Maillard reaction potential, EDTA and a buffer;

cooling the vascular tissue at a rate and to a temperature such that the formation of ice crystals is substantially prevented so as to give a frozen vascular tissue;

drying the frozen vascular tissue by molecular distillation drying.

18. A process of preserving a collagen based nerve tissues for transplantation, the process comprising:

procuring the collagen based nerve tissue from a donor;

immersing the nerve tissue in a stabilization solution,

immersing the nerve tissue in a first detergent solution, wherein the first detergent solution includes a n-octyl-ß-D-glucopyranoside and sodium chloride for solubilizing lipid membranes and proteins, ethylenediamine tetraacetate (EDTA) and a buffer;

washing the nerve tissue with a wash solution, the wash solution including EDTA and buffer;

immersing the nerve tissue in a second detergent solution, wherein the second detergent solution includes octanoic acid, a sodium phosphate buffer and optionally antibiotics;

immersing the nerve tissue in an enzyme solution, wherein the enzyme solution includes DNaseI, sodium chloride and magnesium chloride and a buffer;

immersing the nerve tissue in a second vitrification solution maltodextrine (VSMD) wherein the first VSMD includes: maltodextrin having a low Maillard reaction potential, EDTA and a buffer;

cooling the nerve tissue at a rate and to a temperature such that the formation of ice crystals is substantially prevented so as to give a frozen nerve tissue;

drying the frozen nerve tissue by molecular distillation drying.

INTERNATIONAL SEARCH REPORT

Inte .onal Application No
PCT/US 99/03667

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 564 786 A (LIFECELL CORP) 13 October 1993 see: page 6, line 20 - 37; page 7, line 26 - page 9, line 19; examples 3, 5. ---	1-9
Y	WO 96 32905 A (ST JUDE MEDICAL; BISHOPRIC NANETTE H (US); DOUSMAN LINDA (US); YAO) 24 October 1996 see: page 6, line 27 - page 8, line 4; page 13, line 22 - 31; page 15, line 15 - 24. ---	1-9
Y	EP 0 128 706 A (UNIVERSITY PATENTS INC) 19 December 1984 see page 11, line 10 - page 12, line 28 --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 June 1999	24/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Klaver, J

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INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 99/03667

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 24873 A (CRYOLIFE INC) 21 September 1995 see: page 9, line 25 - page 10, line 3; page 11, line 3 - page 14, line 18. -----	1-9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte :onal Application No
PCT/US 99/03667

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